CERTIFICATION OF TRANSLATION

The undersigned, Jessica T. Abreu, whose address is 2334 N. Van Buren Court, Arlington, VA 22205-1939, USA, declares and states as follows:

I am well acquainted with the English and French languages; I have in the past translated numerous French documents of legal and/or technical content into English; and I am certified by Georgetown University and accredited by the American Translators Association in French into English translation.

I have been asked to translate and/or review a 21-page French patent application, containing 3 additional pages of figures, with a national registration number of **93 08596**, filed on **July 13, 1993**, and entitled: **"VECTEURS VIRAUX ET UTILISATION EN THÉRAPIE GÉNIQUE."**

I hereby declare that the attached translation of the document referenced above is, to the best of my knowledge and ability, a true and accurate translation of the original French document.

And I declare further that all statements made herein of my own knowledge are true, that all statements made on information and belief are believed to be true, and that falsification of these statements and the like is punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code.

I therefore attach my Certification of Translation to the English translation of this document.

November 20, 2001
Date

Jessica T. Abreu

City/County of Arlington
Commonwealth of Virgina
The foregoing instrument was subscribed and sworn to before me on this
20th day of November 2001by

Notary Public

My commission expires on: 03/31/04

VIRAL VECTORS AND USE IN GENE THERAPY

The present invention relates to novel viral vectors, their preparation and use in gene therapy. It also relates to pharmaceutical compositions containing said viral vectors. More particularly, the present invention relates to recombinant adenoviruses as vectors for gene therapy.

Gene therapy consists in correcting a deficiency or an abnormality (mutation, aberrant expression and the like) by introducing genetic information into the cell or affected organ. This genetic information can be introduced either in vitro, in a cell extracted from the organ, with the modified cell then being reintroduced into the body, or directly in vivo into the appropriate tissue. In this second case, various techniques exist, among which are various transfection techniques involving DNA and DEAE-dextran complexes (Pagano et al., J. Virol. 1 (1967) 891), DNA and nuclear protein complexes (Kaneda et al., Science 243 (1989) 375), DNA and lipid complexes (Felgner et al., PNAS 84 (1987) 7413), the use of liposomes (Fraley et al., J. Biol. Chem. 255 (1980) 10431) and the like. More recently, the use of viruses as vectors for the transfer of genes has appeared as a promising alternative to these physical transfection techniques. In this respect, various viruses have been tested for their capacity to infect certain cell populations, in particular, retroviruses (RSV, HMS, MMS and the like), the HSV virus, adeno-associated viruses and adenoviruses.

Among these viruses, adenoviruses present some advantageous properties for use in gene therapy. In particular, they have a fairly broad host spectrum, are capable of infecting quiescent cells, do not integrate into the genome of the infected cell, and have not been associated to date with major pathologies in man.

Adenoviruses are viruses with linear double-stranded DNA of a size of about 36 kb. In particular, their genome comprises an inverted terminal repeat sequence (ITR), an encapsidation sequence, early genes and late genes (cf Figure 1). The principal early genes are the E1 (E1a and E1b), E2, E3 and E4 genes. The principal late genes are the L1 to L5 genes.

Due to the abovementioned properties, adenoviruses

have already been used for the transfer of genes in vivo. To this end, various vectors derived from adenoviruses have been prepared, incorporating various genes (ß-gal, OTC, α-1AT, cytokines and the like). In each of these constructs, the adenovirus was modified so as to render it incapable of replicating in the infected cell. Thus, the constructs described in the prior art are adenoviruses from which the E1 (E1a and/or E1b) and optionally E3 regions have been deleted at the point at which the heterologous DNA sequences are inserted (Levrero et al., Gene 101 (1991) 195; Gosh-Choudhury et al., Gene 50 (1986) 161). Nevertheless, the vectors described in the prior art have numerous disadvantages that limit their exploitation in gene therapy. In particular, all of these vectors contain numerous viral genes whose expression in vivo is not desirable in the context of gene therapy. Furthermore, these vectors do not allow the incorporation of very large DNA fragments, which may be necessary for certain applications.

The present invention makes it possible to overcome these disadvantages. The present invention describes recombinant adenoviruses for gene therapy, which are capable of efficiently transferring DNA (up to 30 kb) in vivo, of expressing this DNA in vivo at high levels and in a stable manner, while limiting any risk of producing viral proteins, of transmitting the virus, or of pathogenicity and the like. In particular, it was found that it is possible to considerably reduce the size of the adenovirus genome without preventing the formation of an encapsidated viral particle. This is surprising since it had been observed in the case of other viruses, such as retroviruses, for example, that certain sequences distributed along the genome were necessary for efficient encapsidation of the viral particles. As such, the production of vectors with substantial internal deletions was very limited. The present invention also shows that suppressing most of the viral genes does not prevent the formation of such a viral particle. Furthermore, recombinant adenoviruses obtained in this manner preserve their advantageous properties of high infectivity, stability in vivo and the like, despite substantial modifications to their genomic structure.

The vectors of the invention are particularly advantageous since they make it possible to incorporate desired DNA sequences of very large sizes. It is thus possible to insert a gene of a length greater than 30 kb. This is particularly advantageous for some pathologies whose treatment requires
the co-expression of several genes, or the expression of very large genes. Thus, for

example, in the case of muscular dystrophy, it had not until now been possible to transfer the cDNA corresponding to the native gene responsible for this pathology (dystrophin gene) because of its large size (14 kb).

The vectors of the invention are also very advantageous since they possess very few functional viral regions and since, as a result, the risks inherent in the use of viruses as vectors in gene therapy such as immunogenicity, pathogenicity, transmission, replication, recombination and the like, are substantially reduced or even suppressed.

The present invention thus provides viral vectors, which are particularly suitable for the transfer and expression in vivo of desired DNA sequences.

A first subject of the present invention therefore relates to a defective recombinant adenovirus comprising:

- ITR sequences,
- a sequence permitting encapsidation,
- a heterologous DNA sequence,

and in which:

- the E1 gene is non-functional and
- at least one of the E2, E4 and L1-L5 genes is non-functional.

For the purposes of the present invention, the term "defective adenovirus" designates an adenovirus that is incapable of replicating autonomously in the target cell. Generally, the genome of defective adenoviruses according to the present invention is therefore devoid of at least the sequences necessary for replication of said virus in the infected cell. These regions can be removed (completely or partially), rendered non-functional, or replaced by other sequences, and in particular by the heterologous DNA sequence.

The inverted terminal repeat sequences (ITR) constitute the replication origin of adenoviruses. They are located at the 3' and 5' ends of the viral genome (cf Figure 1), from where they can easily be isolated according to conventional molecular biology techniques known to persons skilled in the art. The nucleotide sequence of the human adenovirus ITR sequences (in particular of the Ad2 and Ad5 serotypes) is described in the literature, as is that of canine adenoviruses (particularly CAV1 and CAV2).

As regards the Ad5 adenovirus for example, the left ITR sequence corresponds to the region comprising nucleotides 1 to 103 of the genome.

The encapsidation sequence (also designated Psi sequence) is necessary for encapsidation of viral DNA. This region must therefore be present in order to make it possible to prepare defective recombinant adenoviruses according to the invention. The encapsidation sequence is located in the adenovirus genome, between the left (5') ITR and the E1 gene (cf Figure 1). This sequence can be isolated or synthesized artificially by conventional molecular biology techniques. The nucleotide sequence of the human adenovirus encapsidation sequence (in particular of the Ad2 and Ad5 serotypes) is described in the literature, as is that of canine adenoviruses (in particular CAV1 and CAV2). As regards the Ad5 adenovirus for example, the encapsidation sequence corresponds to the region comprising nucleotides 194 to 358 of the genome.

There are various adenovirus serotypes whose structure and properties vary somewhat. Nonetheless, these viruses exhibit comparable genetic organization, and persons skilled in the art can easily reproduce the teachings described in the present application for any type of adenovirus.

The adenoviruses of the invention may be of human, animal or mixed (human and animal) origin.

As regards adenoviruses of human origin, the use of those classified in group C is preferred. More preferably, among the various human adenovirus serotypes, the use of type 2 or 5 adenoviruses (Ad2 or Ad5) is preferred in the context of the present invention.

As indicated above, the adenoviruses of the invention may also be of animal origin, or contain sequences derived from adenoviruses of animal origin. The applicant has indeed shown that adenoviruses of animal origin are capable of infecting human cells very efficiently, and that they are incapable of propagating in the human cells in which they were tested (cf Application FR 93 05954). The applicant has also shown that adenoviruses of animal origin are not at all transcomplemented by adenoviruses of human origin. This eliminates any risk of recombination and propagation in vivo in the presence of a human adenovirus, which could in turn lead to the formation of infectious particles. The use of adenoviruses or adenovirus regions of animal origin is

therefore particularly advantageous since the risks inherent in the use of viruses as vectors in gene therapy are even smaller.

The adenoviruses of animal origin that can be used in the context of the present invention may be of canine, bovine, murine, (e.g., Mavl, Beard et al., Virology 75 (1990) 81), ovine, porcine or avian or alternatively simian origin (e.g., SAV). More particularly, among the avian adenoviruses, mention may be made of serotypes 1 to 10 which are available at ATCC, such as for example the Phelps (ATCC VR-432), Fontes (ATCC VR-280), P7-A (ATCC VR-827), IBH-2A (ATCC VR-828), J2-A (ATCC VR-829), T8-A (ATCC VR-830), K-11 (ATCC VR-921) strains, or alternatively the strains referenced as ATCC VR-831 to 835. Among the bovine adenoviruses, the various known serotypes can be used, and in particular those available at ATCC (types 1 to 8) under references ATCC VR-313, 314, 639-642, 768 and 769. Mention may also be made of murine adenoviruses FL (ATCC VR-550) and E20308 (ATCC VR-528), type 5 (ATCC VR-1343), or type 6 (ATCC VR-1340) ovine adenovirus; porcine adenovirus 5359), or the simian adenoviruses such as in particular the adenoviruses referenced at ATCC under the numbers VR-591-594, 941-943, 195-203 and the like.

Preferably, among the various adenoviruses of animal origin, adenoviruses or adenovirus regions of canine origin, and particularly all of the CAV2 adenovirus strains [Manhattan or A26/61 strain (ATCC VR-800), for example] are used in the context of the invention. Canine adenoviruses have been the subject of numerous structural studies. As such, complete restriction maps of the CAV1 and CAV2 adenoviruses have been described in the prior art (Spibey et al., J. Gen. Virol, 70 (1989) 165), and the E1a and E3 genes, as well as the ITR sequences have been cloned and sequenced (see in particular Spibey et al., Virus Res. 14 (1989) 241; Linné, Virus Res. 23 (1992) 119, WO 91/11525).

As indicated above, the adenoviruses of the present invention contain a heterologous DNA sequence. The heterologous DNA sequence designates any DNA sequence introduced into the recombinant virus, whose transfer and/or expression in the target cell is desired.

In particular, the heterologous DNA sequence may contain one or more therapeutic genes and/or one or more genes encoding antigenic peptides.

The therapeutic genes that can thus be transferred are any genes whose transcription and optionally translation in the target cell generate products that have a therapeutic effect.

In particular, this may be a gene encoding protein products that have a therapeutic effect. The protein product thus encoded may be a protein, a peptide, an amino acid and the like. This protein product may be homologous with respect to the target cell (that is to say a product that is normally expressed in the target cell when the latter presents no pathology). In this case, the expression of a protein makes it possible, for example, to palliate an insufficient expression in the cell, or the expression of a protein which is inactive or weakly active as a result of a modification, or alternatively to overexpress said protein. The therapeutic gene may also encode a mutant of a cellular protein, with increased stability, modified activity and the like. The protein product may also be heterologous with respect to the target cell. In this case, an expressed protein can, for example, supplement or provide an activity that is deficient in the cell, enabling it to combat a pathology.

Among the therapeutic products defined in the present invention, more particular mention may be made of enzymes, blood derivatives, hormones, lymphokines: interleukins, interferons, TNF, and the like (FR 9203120), growth factors, neurotransmitters or their precursors or synthetic enzymes, trophic factors: BDNF, CNTF, NGF, IGF, GMF, aFGF, bFGF, NT3, NT5 and the like; apolipoproteins: ApoAI, ApoAIV, ApoE and the like (FR 93 05125), dystrophin or minidystrophin (FR 9111947), tumor suppressor genes: p53, Rb, Rap1A, DCC, k-rev and the like (FR 93 04745), the genes encoding factors involved in coagulation: Factors VII, VIII, IX and the like.

The therapeutic gene can also be an antisense gene or sequence, whose expression in the target cell makes it possible to control the expression of genes or the transcription of cellular mRNAs. Such sequences can, for example, be transcribed in the target cell into RNAs that are complementary to cellular mRNAs, and thus block their translation into protein, according to the technique described in Patent document EP 140 308.

As indicated above, the heterologous DNA sequence may also contain one or more genes encoding an antigenic peptide, which is capable of generating an immune response in man. In this particular embodiment, the invention therefore permits the production of vaccines, which make it possible to immunize

man, especially against microorganisms or viruses. In particular, these may be antigenic peptides specific to the Epstein-Barr virus, the HIV virus, the hepatitis B virus (EP 185 573), the pseudo-rabies virus, or alternatively specific to tumors (EP 259 212).

Generally, the heterologous DNA sequence also comprises sequences permitting the expression of the therapeutic gene and/or of the gene encoding the antigenic peptide in the infected cell. These may be sequences that are naturally responsible for the expression of the gene in question, when these sequences are capable of functioning in the infected cell. They may also be sequences of a different origin (sequences which are responsible for the expression of other proteins, or even synthetic sequences). In particular, they may be eukaryotic or viral gene promoter sequences. For example, they may be promoter sequences derived from the genome of the cell of which infection is desired. Likewise, they may be promoter sequences derived from the genome of a virus, including the adenovirus used. In this respect, mention may be made for example of the E1A, MLP, CMV and RSV gene promoters and the like. In addition, adding activating sequences, regulatory sequences and the like can modify these expression sequences. Moreover, when the inserted gene does not contain expression sequences, it can be inserted into the genome of the defective virus downstream of such a sequence.

Furthermore, the heterologous DNA sequence may also contain, particularly upstream of the therapeutic gene, a signal sequence directing the therapeutic product synthesized in the secretory pathways of the target cell. This signal sequence may be the natural signal sequence of the therapeutic product, but it may also be any other functional signal sequence, or an artificial signal sequence.

As indicated above, the vectors of the invention possess at least one of the non-functional E2, E4 and L1-L5 genes. The viral gene in question can be rendered non-functional by any technique known to a person skilled in the art, and in particular by suppressing, substituting deleting or adding one or more bases to the gene(s) in question. Such modifications can be obtained in vitro (on isolated DNA) or in situ, for example, by means of genetic engineering techniques, or alternatively by treatment with mutagenic agents.

Among the mutagenic agents, mention may be made, for example, of physical agents such as energetic radiation (X-, g- and ultraviolet rays and the like), or

chemical agents which are capable of reacting with various functional groups of DNA bases, and for example alkylating agents [ethyl methanesulphonate (EMS), N-methyl-N'-nitro-N-nitrosoguanidine, N-nitroquinoline-1-oxide (NQO)], bialkylating agents, intercalating agents and the like.

For purposes of the invention, "deletion" is understood to be any suppression of the gene in question. In particular, this may be all or part of the coding region of said gene, and/or all or part of the promoter region for transcription of said gene. The suppression can be carried out by digestion via appropriate restriction enzymes, and then ligation, according to conventional molecular biology techniques, as illustrated in the examples.

The genetic modifications can also be obtained by gene disruption, for example according to the protocol initially described by Rothstein [Meth. Enzymol. 101 (1983) 202]. In this case, all or part of the coding sequence is preferably perturbed so as to permit replacement of the genomic sequence by a non-functional or mutant sequence via homologous recombination,

The aforementioned genetic modification(s) may be located in the coding part of the relevant gene, or outside the coding region, and, for example, in the regions responsible for the expression and/or transcriptional regulation of said genes. The non-functional character of these genes can therefore manifest by production of an inactive protein due to structural or conformational modifications, by the absence of production, by the production of a protein with altered activity, or alternatively by the production of the natural protein at an attenuated level or according to a desired mode of regulation.

Moreover, some alterations, such as point mutations, are by nature capable of being corrected or attenuated by cellular mechanisms. Such genetic alterations are then of limited interest for industrial purposes. It is therefore particularly preferred that the non-functional character be perfectly segregationally stable and/or non-reversible.

Preferably, the gene is non-functional because of a partial or total deletion.

Preferably, the defective recombinant adenoviruses of the invention are devoid of adenovirus late genes.

A particularly advantageous embodiment of the invention consists in a defective recombinant adenovirus comprising:

- the ITR sequences,
- a sequence permitting encapsidation,
- a heterologous DNA sequence, and
- a region carrying the E2 gene or a part of the E2 gene.

Another particularly advantageous embodiment of the invention consists in a defective recombinant adenovirus comprising:

- the ITR sequences,
- a sequence permitting encapsidation,
- a heterologous DNA sequence, and
- a region carrying the E4 gene or a part of the E4 gene.

Still in a particularly advantageous embodiment, the vectors of the invention possess, in addition, a functional E3 gene under the control of a heterologous promoter. More preferably, the vectors possess part of the E3 gene, permitting the expression of the gp 19K protein.

Defective recombinant adenoviruses according to the invention can be prepared in various ways.

A first method consists in transfecting the DNA from the defective recombinant virus prepared in vitro (either by ligation, or in plasmid form) into a competent cell line, that is to say carrying in trans all of the functions necessary for the complementation of the defective virus. These functions are preferably integrated into the genome of the cell, which makes it possible to avoid the risks of recombination, and confers increased stability on the cell line. The preparation of such cell lines is described in the examples.

A second approach consists in co-transfecting the DNA from the defective recombinant virus prepared in vitro (either by ligation, or in plasmid form) and the DNA from a helper virus into an appropriate cell line. According to this method, it is not necessary to have a competent cell line capable of complementing all of the defective functions of the recombinant adenovirus. Part of these functions is indeed complemented by the helper virus. This helper virus must itself be defective and the cell line carries in trans the functions necessary for its complementation. The preparation of defective recombinant adenoviruses of the invention according to this method is also illustrated in the examples.

Among the cell lines which can be used in this second approach, particular mention may be made of the human embryonic kidney cell line 293, the KB cells, the Hela cells, the MDCK cells, the GHK cells and the like (cf examples).

Then the vectors, which have multiplied, are recovered, purified and amplified according to conventional molecular biology techniques.

The present invention therefore also relates to cell lines which can be infected by adenoviruses, comprising, integrated into their genome, the functions necessary for the complementation of a defective recombinant adenovirus as described above. In particular, it relates to cell lines containing the E1 and E2 regions (particularly the region encoding the 72K protein) and/or E4 and/or the gene for the glucocorticoid receptor integrated into their genome. Preferably, these lines are obtained from the 293 or gm DBP6 cell line.

The present invention also relates to any pharmaceutical composition comprising one or more defective recombinant adenoviruses as described above. The pharmaceutical compositions of the invention can be formulated for topical, oral, parenteral, intranasal, intravenous, intramuscular, subcutaneous, intraocular and transdermal administration and the like.

Preferably, the pharmaceutical composition contains vehicles that are pharmaceutically acceptable for injectable formulations. In particular, these may be saline (monosodium or disodium phosphate, sodium, potassium, calcium or magnesium chloride and the like or mixtures of such salts), sterile or isotonic solutions, or dry, particularly freeze-dried, compositions, which by addition of sterilized water or sterile saline, as the case may be, permit the constitution of injectable solutions.

The virus doses used for the injection can be adapted according to various parameters, and particularly with a view to the mode of administration used, the relevant pathology, the gene to be expressed, or alternatively the desired duration of treatment. Generally, recombinant adenoviruses according to the invention are formulated and administered in the form of doses of between 10^4 and 10^{14} pfu/ml, and preferably 10^6 to 10^{10} pfu/ml. The term pfu ("plaque forming unit") corresponds to the infectivity of a virus solution, and is determined by infecting an appropriate cell culture, and measuring, generally after 5 days, the number of plaques of

infected cells. The techniques for determining the pfu titer of a viral solution are well documented in the literature.

Depending on the inserted heterologous DNA sequence, the adenoviruses of the invention can be used for the treatment or prevention of numerous pathologies including genetic diseases (dystrophy, cystic fibrosis and the like), neurodegenerative diseases (Alzheimer's, Parkinson, ALS and the like), cancers, pathologies linked to coagulation disorders and to dyslipoproteinaemias, pathologies linked to viral infections (hepatitis, AIDS and the like) and the like.

The present invention will be more fully described in the following examples, which are to be considered as illustrative and non-limiting.

Legend to the figures

Figure 1: Genetic organization of the Ad5 adenovirus. The complete sequence of Ad5 is available on database and enables persons skilled in the art to select or create any restriction site, and thus isolate any region of the genome.

Figure 2: Restriction map of the CAV2 adenovirus Manhattan strain (according to Spibey et al., previously cited).

Figure 3: Construction of defective viruses of the invention by ligation.

Figure 4: Construction of a recombinant virus carrying the E4 gene.

Figure 5: Construction of a recombinant virus carrying the E2 gene.

General molecular biology techniques

The conventional methods used in molecular biology such as preparative extractions of plasmid DNA, centrifugation of plasmid DNA in cesium chloride gradient, electrophoresis on agarose or acrylamide gels, purification of DNA fragments by electroelution, protein extractions with phenol or phenol-chloroform, DNA precipitation in saline medium with
ethanol or isopropanol, transformation in Escherichia coli and the like, are well known to persons skilled in the art and are widely described in the literature [Maniatis T. et al., "Molecular Cloning, a Laboratory Manual", Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1982; Ausubel F.M. et al. (eds), "Current Protocols in Molecular Biology", John Wiley & Sons, New York, 1987].

The pBR322- and pUC-type plasmids and the phages of the M13 series are of commercial origin (Bethesda Research Laboratories).

For the ligations, the DNA fragments can be separated according to their size by agarose or acrylamide gel electrophoresis, extracted with phenol or with a phenol/chloroform mixture, precipitated with ethanol and then incubated in the presence of phage T4 DNA ligase (Biolabs) according to the supplier's recommendations.

The protruding 5' ends can be filled with the E. Coli Polymerase I DNA Klenow fragment (Biolabs) according to the supplier's specifications. The protruding 3' ends are destroyed in the presence of phage T4 DNA Polymerase (Biolabs), which is used according to the manufacturer's recommendations. The protruding 5' ends are destroyed by controlled treatment with S1 nuclease.

Site-directed mutagenesis in vitro with synthetic oligodeoxynucleotides can be carried out according to the method developed by Taylor et al. [Nucleic Acids Res. 13 (1985) 8749-8764] using the kit distributed by Amersham.

Enzymatic amplification of DNA fragments by the so-called PCR technique [Polymerase-catalyzed Chain Reaction, Saiki R.K. et al., Science 230 (1985) 1350-1354; Mullis K.B. and Faloona F.A., Meth. Enzym. 155 (1987) 335-350] can be carried out using the "DNA thermal cycler" (Perkin Elmer Cetus) according to the manufacturer's specifications.

Verification of the nucleotide sequences can be carried out using the method developed by Sanger et al. [Proc. Natl. Acad. Sci. USA, 74 (1977) 5463-5467] using the kit distributed by Amersham.

Cell lines used

In the following examples, the following cell lines were or can be used:

- Human embryonic kidney line 293 (Graham et al., J. Gen. Virol. 36 (1977) 59). In particular, this cell line contains the left part of the human Ad5 adenovirus genome (12%) integrated into its own genome.

- Human cell line KB: derived from a human epidermal carcinoma, this cell line is available at ATCC (ref. CCL17) as are its culturing conditions.

- Human cell line Hela: derived from a carcinoma of the human epithelium, this cell line is available at ATCC (ref. CCL2) as are its culturing conditions.

- Canine cell line MDCK: the conditions for culture of the MDCK cells are described by Macatney et al., Science 44 (1988) 9.

- gm DBP6 cell line (Brough et al., Virology 190 (1992) 624). This cell line consists of Hela cells carrying the adenovirus E2 gene under the control of the MMTV LTR.

EXAMPLES

Example 1

This example demonstrates the feasibility of a recombinant adenovirus devoid of most of the viral genes. For this purpose, a series of adenovirus deletion mutants was constructed by ligation in vitro, and each of these mutants was co-transfected with a helper virus into the KB cells. Since these cells do not allow propagation of the viruses defective for E1, the transcomplementation is directed to the E1 region.

The various deletion mutants were prepared from the Ad5 adenovirus by digestion and then ligation in vitro. For this purpose, the viral DNA from Ad5 is isolated according to the technique described by Lipp et al. (J. Virol. 63 (1989) 5133), subjected to digestion in the presence of various restriction enzymes (cf Figure 3), and then the digestion product is ligated in the presence of T4 DNA ligase. The size of the various deletion mutants is then checked on 0.8% SDS-agarose gel. These mutants are then mapped (cf Figure 3). These various mutants contain the following regions:

mt1: Ligation between the Ad5 fragments 0-20642(SauI) and (SauI)33797-35935

mt2: Ligation between the Ad5 fragments 0-19549(NdeI) and (NdeI)31089-35935

mt3: Ligation between the Ad5 fragments 0-10754(AatII) and (AatII)25915-35935

mt4: Ligation between the Ad5 fragments 0-11311(MluI) and (MluI)24392-35935

mt5: Ligation between the Ad5 fragments 0-9462(SalI) and (XhoI)29791-35935

mt6: Ligation between the Ad5 fragments 0-5788(XhoI) and (XhoI)29791-35935

mt7: Ligation between the Ad5 fragments 0-3665(SphI) and (SphI)31224-35935

Each of the mutants prepared above was co-transfected with the viral DNA from Ad.RSVßGal (Stratford-Perricaudet et al., J. Clin. Invest. 90 (1992) 626) into the KB cells, in the presence of calcium phosphate. The cells were harvested 8

days after transfection, and the culture supernatants were harvested and then amplified on KB cells until stocks of 50 dishes were obtained for each transfection. Episomal DNA was isolated from each sample, and separated on cesium chloride gradient. Two distinct virus bands were observed in each case, and were collected and analyzed. The heavier band corresponds to the viral DNA from Ad.RSVßGal, and the lighter band to the DNA from the recombinant virus generated by ligation (Figure 3). The titer obtained for the latter is about 10^8 pfu/ml.

A second series of adenovirus deletion mutants was constructed by ligation in vitro according to the same methodology. These various mutants contain the following regions:

mt8: Ligation between fragments 0-4623(ApaI) from Ad RSVßGal and (ApaI)31909-35935 from Ad5.

mt9: Ligation between fragments 0-10178(BglII) from Ad RSVßGal and (BamHI)21562-35935 from Ad5.

These mutants, containing the LacZ gene under the control of the LTR promoter of the RSV virus, are then co-transfected into the 293 cells in the presence of the viral DNA from H2d1808 (Weinberg et al., J. Virol. 57 (1986) 833), from which the E4 region is deleted. According to this second technique, the transcomplementation is directed to E4 and is no longer directed to E1. As described above, this technique thus makes it possible to generate recombinant viruses possessing only the E4 region as a viral gene.

Example 2

This example describes the preparation of defective recombinant adenoviruses according to the invention by co-transfection, with a helper virus, of the recombinant virus DNA incorporated into a plasmid.

For this purpose, a plasmid was constructed, carrying the joining Ad5 ITRs, the encapsidation sequence, the E4 gene under the control of its own promoter and the LacZ gene under the control of the RSV virus LTR promoter as a heterologous gene (Figure 4). This plasmid, designated pE4Gal was obtained by cloning and ligation of the following fragments (see Figure 4):

- HindIII-SacII fragment derived from plasmid pFG144 (Graham et al., EMBO J. 8 (1989) 2077). This fragment carries the Ad5 ITR sequences in tandem and the encapsidation sequence : HindIII (34920)-SacII (352) fragment;

- Ad5 fragment contained between the SacII (located at base pair 3827) and PstI (located at base pair 4245) sites;

- pSP 72 (Promega) fragment contained between the PstI (bp 32) and SalI (bp 34) sites;

- XhoI-XbaI fragment of plasmid pAdLTR GalIX described in Stratford-Perricaudet et al. (JCI 90 (1992) 626). This fragment carries the LacZ gene under the control of the RSV virus LTR;

- XbaI (bp 40) - NdeI (bp 2379) fragment of plasmid pSP 72;

- NdeI (bp 31089) - HindIII (bp 34930) fragment from Ad5. This fragment, located in the right end of the Ad5 genome, contains the E4 region under the control of its own promoter. It was cloned into the NdeI site (2379) of plasmid pSP 72 and HindIII site of the first fragment.

This plasmid was obtained by cloning the various fragments into the indicated regions of plasmid pSP 72. It is understood that equivalent fragments can be obtained by persons skilled in the art from other sources.

Plasmid pE4Gal is then co-transfected with the DNA from virus H2dl808 into the 293 cells in the presence of calcium phosphate. The recombinant virus is then prepared as described in Example 1. This virus carries the E4 gene from the Ad5 adenovirus as its sole viral gene (Figure 4). The size of its genome is approximately 12 kb, which makes the insertion of very large heterologous DNA (up to 20 kb) possible. Thus, persons skilled in the art can easily replace the LacZ gene with any other therapeutic gene such as those mentioned above. Moveover, this virus contains some sequences derived from plasmid pSP 72, which can be removed by conventional molecular biology techniques if necessary.

Example 3

This example describes the preparation of another defective recombinant adenovirus according to the invention, by co-transfection, with a helper virus, of the recombinant virus DNA incorporated into a plasmid.

For this purpose, a plasmid was constructed carrying the joined Ad5 ITRs, the encapsidation sequence, the Ad2 E2 gene under the control of its own promoter and the LacZ gene under the control of the RSV virus LTR promoter as a heterologous gene (Figure 5). This plasmid, designated pE2Gal was obtained by cloning and ligation of the following fragments (see Figure 5):

- HindIII-SacI fragment derived from plasmid pFG144 (Graham et al., EMBO J.8 (1989) 2077). This fragment carries the Ad5 ITR sequences in tandem and the encapsidation sequence: HindIII (34920)-SacII (352) fragment. It was cloned with the following fragment into the HindIII(16)-PstI(32) sites of plasmid pSP 72;

- Ad5 fragment contained between the SacII (located at base pair 3827) and PstI (located at base pair 4245) sites. This fragment was cloned into the SacII site of the preceding fragment and the PstI (32) site of plasmid pSP 72;

- pSP 72 (Promega) fragment contained between the PstI (bp 32) and SalI (bp 34) sites;

- XhoI-XbaI fragment of plasmid pAdLTR GalIX described in Stratford-Perricaudet et al. (JCI 90(1992)626). This fragment carries the LacZ gene under the control of the RSV virus LTR. It was cloned into the SalI(34) and XbaI sites of plasmid pSP 72.

- pSP 72 (Promega) fragment contained between the XbaI(bp 34) and BamHI(bp 46) sites;

- BamHI(bp 21606) - SmaI(bp 27339) fragment of Ad2. This fragment of the Ad2 genome contains the E2 region under the control of its own promoter. It was cloned into the BamHI(46) and EcoRV sites of plasmid pSP 72;

- EcoRV(bp 81) - HindIII(bp 16) fragment of plasmid pSP 72.

This plasmid was obtained by cloning the various fragments into the indicated regions of plasmid pSP 72. It is understood that equivalent fragments can be obtained by persons skilled in the art from other sources.

Plasmid pE2Gal is then co-transfected with the DNA from the H2d1802 virus devoid of the E2 region (Rice et al. J. Virol. 56(1985)767) into the 293 cells, in the presence of calcium phosphate. The recombinant virus is then prepared as described in Example 1. This virus carries, as its sole viral gene, the E2 gene from the Ad2 adenovirus (Figure 5). The size of its genome is approximately 12 kb, which makes the insertion of very large heterologous DNA (up to 20 kb) possible. Thus, persons skilled in the art can easily replace the LacZ gene with any other therapeutic gene such as those mentioned above. Moreover, this virus contains some sequences derived from the intermediate plasmid, which can be removed by conventional molecular biology techniques if necessary.

Example 4

This example describes the construction of complementing cell lines for the E1, E2 and/or E4 regions of adenoviruses. These cell lines permit the construction of recombinant E1, E2 and/or E4 region-deleted adenoviruses according to the invention without the use of a helper virus. These viruses are obtained by in vivo recombination, and may contain major heterologous sequences.

In the cell lines described, the E2 and E4 regions, which are potentially cytotoxic, are placed under the control of an inducible promoter: the MMTV LTR (Pharmacia) which is induced by dexamethasone. It is understood that other promoters can be used, and particularly MMTV LTR variants which, for example, carry heterologous regulatory regions (particularly an "enhancer" region). The cell lines of the invention were constructed by transfecting the corresponding cells, in the presence of calcium phosphate, with a DNA fragment carrying the indicated genes (adenovirus regions and/or the gene for the glucocorticoid receptor) under the control of a transcription promoter and a terminator (polyadenylation site). The terminator may be either the natural terminator of the transfected gene, or a different terminator such as, for example, the early messenger terminator of the SV40 virus. Advantageously, the DNA fragment also carries a gene allowing selection of the transformed cells, and, for example, the gene for resistance to geneticin. The resistance gene can also be carried by a different DNA fragment, co-transfected with the first.

After transfection, the transformed cells are selected and their DNA is analyzed in order to verify the integration of the DNA fragment into the genome.

This technique makes it possible to obtain the following cell lines:

1. 293 cells having the 72K gene from the Ad5 E2 region under the control of the MMTV LTR;

2. 293 cells having the 72K gene from the Ad5 E2 region under the control of the MMTV LTR and the glucocorticoid receptor gene;

3. 293 cells having the 72K gene from the Ad5 E2 region under the control of the MMTV LTR and the E4 region under the control of the MMTV LTR;

4. 293 cells having the 72K gene from the Ad5 E2 region under the control of the MMTV LTR, the E4 region under the control of the MMTV LTR and the glucocorticoid receptor gene;

5. 293 cells having the E4 region under the control of the MMTV LTR;

6. 293 cells having the E4 region under the control of the MMTV LTR and the glucorticoid receptor gene;

7. gm DBP6 cells having the E1A and E1B regions under the control of their own promoter;

8. gm DBP6 cells having the E1A and E1B regions under the control of their own promoter and the E4 region under the control of the MMTV LTR.

CLAIMS

1. Defective recombinant adenovirus comprising:

- the ITR sequences,

- a sequence permitting encapsidation,

- a heterologous DNA sequence,

and in which the E1 gene and at least one of the E2, E4 and L1-L5 genes is non-functional.

2. Adenovirus according to Claim 1, characterized in that it is of human, animal or mixed origin.

3. Adenovirus according to Claim 2, characterized in that the adenoviruses of human origin are chosen from those classified in group C, preferably from type 2 or 5 adenoviruses (Ad2 or Ad5).

4. Adenovirus according to Claim 2, characterized in that the adenoviruses of animal origin are chosen from adenoviruses of canine, bovine, murine, ovine, porcine, avian or simian origin.

5. Adenovirus according to one of the preceding claims, characterized in that it is devoid of late genes.

6. Adenovirus according to Claim 1, characterized in that it comprises:

- the ITR sequences

- a sequence permitting encapsidation,

- a heterologous DNA sequence, and

- a region carrying the E2 gene or part of the E2 gene.

7. Adenovirus according to Claim 1, characterized in that it comprises:

- the ITR sequences,

- a sequence permitting encapsidation,

- a heterologous DNA sequence, and

- a region carrying the E4 gene or part of the E4 gene.

8. Adenovirus according to one of the preceding claims, characterized in that it comprises, in addition, a functional E3 gene under the control of a heterologous promoter.

9. Adenovirus according to one of the preceding claims, characterized in that the heterologous DNA sequence contains one or more therapeutic genes and/or one or more genes encoding antigenic peptides.

10. Adenovirus according to Claim 9, characterized in that the therapeutic gene is chosen from the genes encoding enzymes, blood derivatives, hormones, lymphokines (interleukins, interferons, TNF and the like), growth factors, neurotransmitters or their precursors or synthetic enzymes, trophic factors (BDNF, CNTF, NGF, IGF, GMF, aFGF, bFGF, NT3, NT5 and the like), apolipoproteins (ApoAI, ApoAIV, ApoE and the like), dystrophin or a minidystrophin, tumor suppressor genes or genes encoding factors involved in coagulation (Factors VII, VIII, IX and the like).

11. Adenovirus according to Claim 9, characterized in that the therapeutic gene is an antisense gene or sequence whose expression in the target cell makes it possible to control the expression of genes or the transcription of cellular mRNAs.

12. Adenovirus according to Claim 9, characterized in that the gene encodes an antigenic peptide capable of generating an immune response in man against microorganisms or viruses.

13. Adenovirus according to Claim 12, characterized in that the gene encodes an antigenic peptide specific for the Epstein Barr virus, the HIV virus, the hepatitis B virus, the pseudo-rabies virus or alternatively specific for tumors.

14. Adenovirus according to one of the preceding claims, characterized in that the heterologous DNA sequence also comprises sequences permitting the expression of the therapeutic gene and/or of the gene encoding the antigenic peptide in the infected cell.

15. Adenovirus according to one of the preceding claims, characterized in that the heterologous DNA sequence comprises, upstream of the therapeutic gene, a signal sequence directing the therapeutic product synthesized in the secretory pathways of the target cell.

16. Cell line infectible by an adenovirus comprising, integrated into its genome, the functions necessary for the complementation of a defective recombinant adenovirus according to one of Claims 1 to 15.

17. Cell line according to Claim 16, characterized in that it contains, in its genome, at least the E1 and E2 genes from an adenovirus.

18. Cell line according to Claim 17, characterized in that it contains, in addition, the E4 gene from an adenovirus.

19. Cell line according to Claim 16, characterized in that it contains, in its genome, at least the E1 and E4 genes from an adenovirus.

20. Cell line according to Claims 17 to 19, characterized in that it contains, in addition, the gene for the glucocorticoid receptor.

21. Cell line according to Claims 17 to 20, characterized in that the E2 and E4 genes are placed under the control of an inducible promoter.

22. Cell line according to Claim 21, characterized in that the inducible promoter is the MMTV LTR promoter.

23. Cell line according to Claims 17 to 22, characterized in that the E2 gene encodes the 72K protein.

24. Cell line according to Claims 16 to 23, characterized in that it is obtained from cell line 293.

25. Pharmaceutical composition comprising at least one defective recombinant adenovirus according to one of Claims 1 to 15.

26. Pharmaceutical composition according to Claim 25, comprising a recombinant adenovirus according to one of Claims 5 to 7.

27. Pharmaceutical composition according to Claims 25 or 26, comprising a vehicle which is pharmaceutically acceptable for an injectable formulation.

Fig.1.

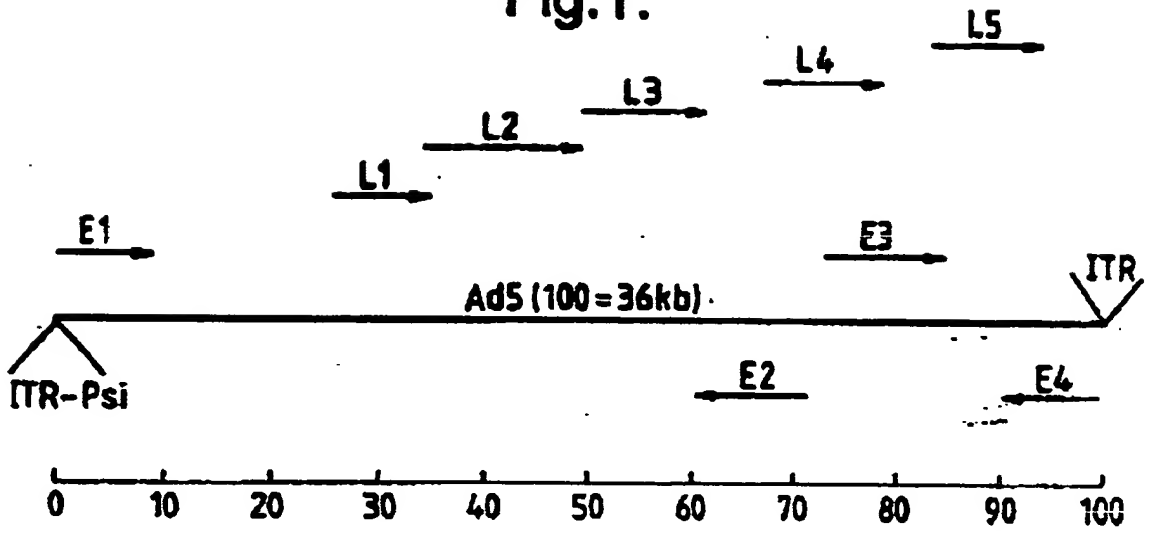
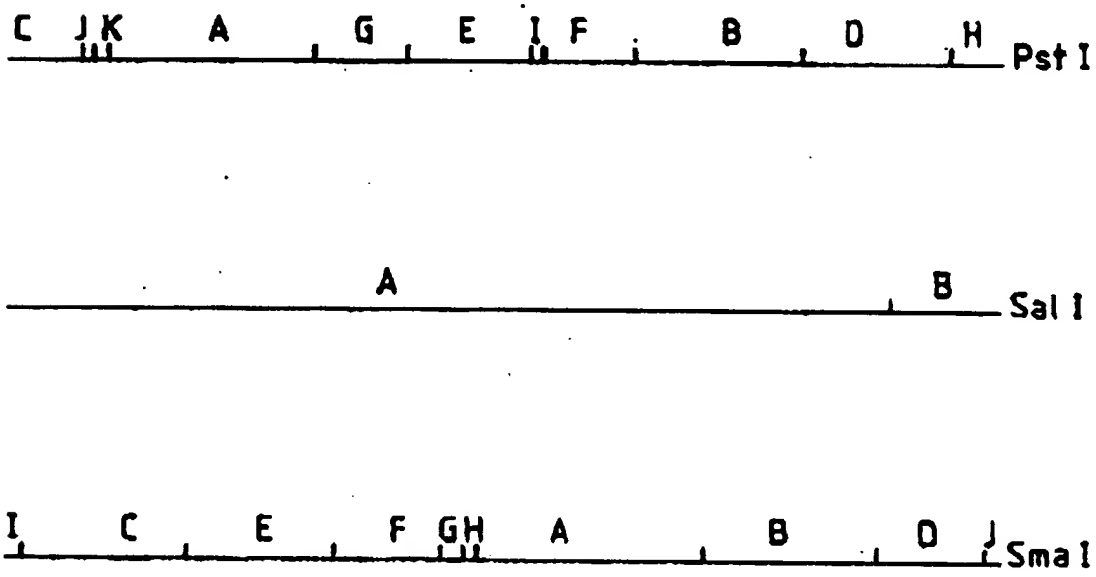

L5
L4
L3
L2
L1
E1
E3
ITR
Ad5 (100=36kb)
ITR-Psi
E2
E4
0
10
20
30
40
50
60
70
80
90
100

Fig.2.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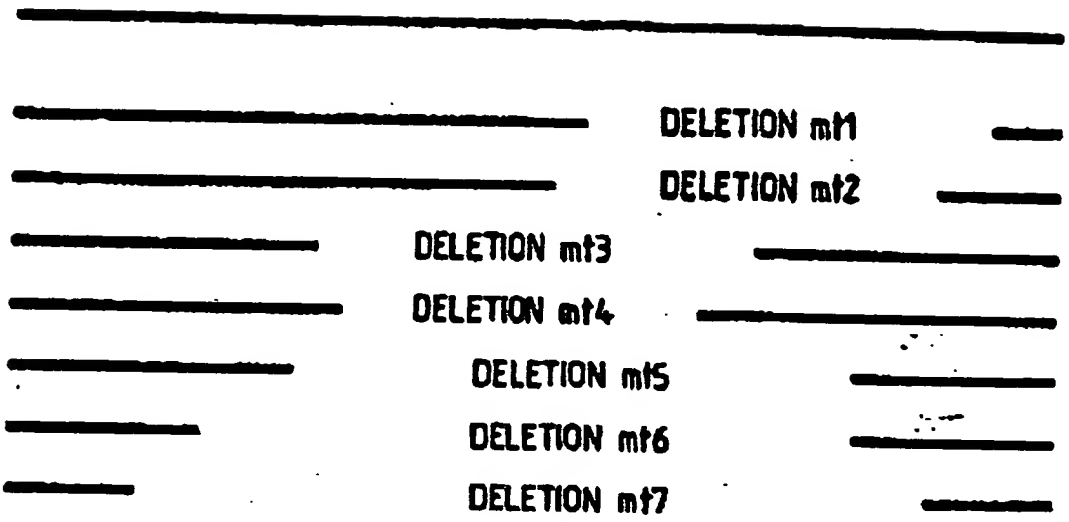
Pst I
A B
Sal I
I C E F G H A B D J
Sma I

Fig.3.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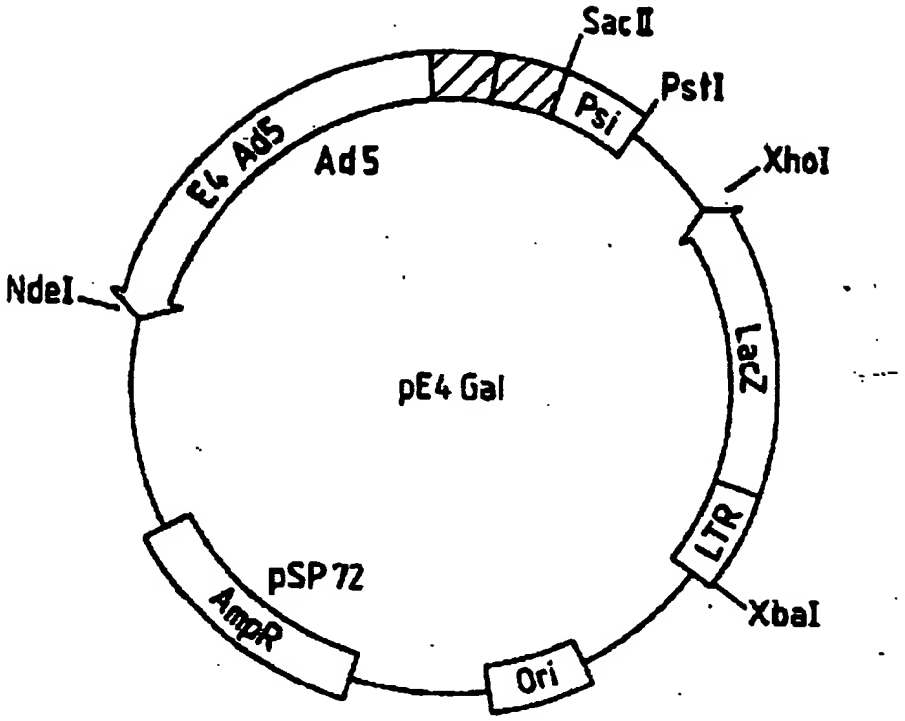
DELETION mt2
DELETION mt3
DELETION mt4
DELETION mt5
DELETION mt6
DELETION mt7

Fig.4.



X

H2d1808

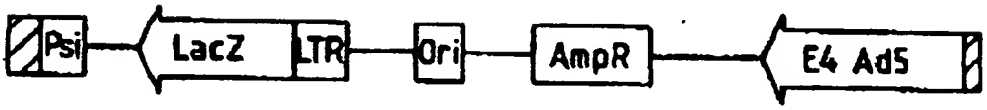
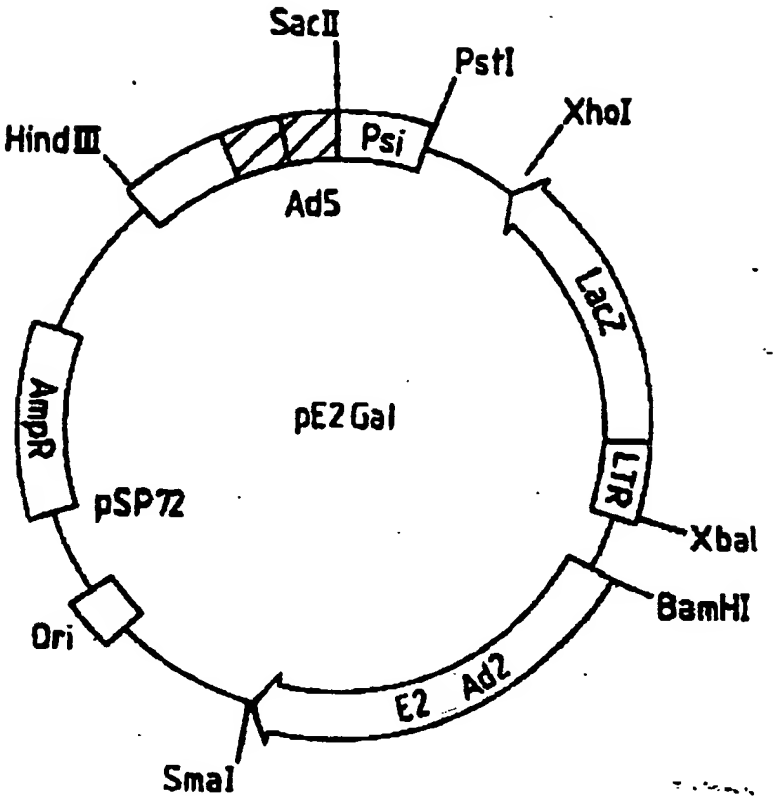


Fig.5.



X

H2d1802